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Inhibition of cellular protein secretion by picornaviral 3A proteins

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Abstract

During poliovirus infection, anterograde traffic between the endoplasmic reticulum and the Golgi is inhibited due to the action of 3A, an 87 amino acid viral protein. The ability of poliovirus protein 3A to inhibit ER-to-Golgi traffic is not required for virus growth. Instead, we have suggested that the inhibition of host protein secretion, shown to reduce the secretion of interferon-β, IL-6, and IL-8 and the expression of both newly synthesized MHC class I and TNF receptor in the plasma membrane of infected cells, affects growth in host organisms. To determine whether the ability of poliovirus 3A to inhibit ER-to-Golgi traffic is conserved, the ability of 3A proteins from several picornaviruses, including human rhinovirus 14, foot-and-mouth disease virus, enterovirus 71, hepatitis A, and Theiler's virus, was tested. Only the 3A proteins from another poliovirus, Sabin 3, and closely related coxsackievirus B3 inhibited ER-to-Golgi traffic as effectively as the 3A protein from poliovirus Mahoney type 1. Site-directed mutagenesis based on these findings and the three-dimensional structure of the amino-terminal domain of poliovirus 3A protein revealed that residues in the unstructured amino terminus of 3A are critical for the inhibition of host protein secretion.

Keywords: 3A proteins; Cellular protein secretion; Picornavirus; Endoplasmic reticulum; Viral protein structure

Introduction

Infection with poliovirus causes the inhibition of host protein secretion by a mechanism independent of the inhibition of other host processes such as translation and nuclear transcription (Doedens and Kirkegaard, 1995). Poliovirus 3A protein can, when expressed in isolation, specifically inhibit ER-to-Golgi traffic in mammalian cells, causing proteins otherwise destined for export to accumulate in ER-derived membranes (Doedens et al., 1997; Neznanov et al., 2001). The 3A-mediated inhibition of protein secretion has been shown to reduce the secretion of antiviral cytokines such as IL-6, IL-8, and interferon-β from poliovirus-infected cells (Dodd et al., 2001), the concentration of TNF receptor on the surface of infected cells (Neznanov et al., 2001), and the presentation of antigens in the context of MHC class I molecules (Deitz et al., 2000). However, the consequences of these effects on viral pathogenesis are not yet known. The inhibition of host protein secretion is not required for virus production per se, because mutant polioviruses that do not inhibit ER-to-Golgi traffic do not show significant growth defects in tissue culture (Bernstein and Baltimore, 1988; Dodd et al., 2001; Doedens et al., 1997).

Poliovirus RNA replication occurs on the cytoplasmic surface of double-membraned vesicles that proliferate during viral infection (Dales et al., 1965; Schlegel et al., 1996; Suhy et al., 2000). All viral proteins required for RNA replication, including 3A and its precursors, are physically localized to these membranes in infected cells (reviewed in Egger et al., 2004). Thus, 3A, its precursors, and the complexes they form are certain to play many roles during viral infection. Mutations in the 3A coding region give rise to viruses defective in RNA synthesis (Giachetti et al., 1992; Teterina et al., 2003) and cell lysis (Lama et al., 1998), but it is not known which biochemical functions of the 3A sequences, or of proteolytic precursors such as 3AB, are disrupted by these mutations. Interestingly, mutations in 3A have been correlated to dramatic changes in pathogenesis in

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host animals for both foot-and-mouth disease virus (Beard and Mason, 2000; Nunez et al., 2001) and hepatitis A virus (Beneduce et al., 1995). The ability of other picornaviruses to inhibit host protein secretion during infection has not yet been tested.

To understand the mechanism of action of poliovirus 3A protein and to apply this knowledge to our understanding of picornaviral pathogenesis, we sought to determine whether the ability of poliovirus 3A protein to inhibit cellular protein secretion was shared with other picornaviruses. We then used this information, in combination with the recently determined three-dimensional structure of the amino-terminal domain of poliovirus 3A (Strauss et al., 2003), to identify new loss-of-function alleles of poliovirus 3A protein that fail to inhibit ER-to-Golgi traffic. Furthermore, mutations could be introduced into the 3A protein of rhinovirus 14 to confer the ability to inhibit host protein secretion.

Results

C-terminally FLAG-tagged 3A proteins do not inhibit secretion, but can be used to monitor protein expression

Our goal was to test whether 3A proteins encoded by a variety of picornaviruses inhibit secretion, as has been shown for poliovirus 3A protein. To this end, various 3A coding regions were amplified by PCR or by RT-PCR. The resulting fragments were inserted into a dicistronic vector with the viral 3A coding region as the first cistron and vesicular stomatitis virus glycoprotein (VSV-G) as the second cistron, under the translational control of the poliovirus IRES (Fig. 1A). Expression of the 87-amino acid 3A proteins from either poliovirus Mahoney type 1 or Sabin type 3 could be confirmed using antibody generated against poliovirus 3A protein encoded by the type 1 genome (Fig. 1D; Doedens et al., 1997). However, since antibodies to other picornaviral 3A proteins were not readily available and, in any case, would not allow determination of the relative abundances of disparate 3A proteins, a second set of plasmids was made that incorporated the FLAG epitope (DYKDDDDK) at the C terminus of each 3A protein (Fig. 1B). For the 3A proteins encoded by polioviruses 1 and 3, addition of the FLAG epitope somewhat reduced the amount of protein that accumulated upon transfection, as could be determined by probing with the anti-3A antibody (Fig. 1D; compare lanes 7 and 9 to lanes 6 and 8). However, detection with the anti-FLAG antibody (Fig. 1E) facilitated direct comparison with the expression of 3A proteins from other picornaviruses; only proteins that could be detected by immunoblotting using an anti-FLAG antibody were included in this study.

To monitor the rate of ER-to-Golgi traffic in the presence and absence of expressed 3A proteins, the Golgi-specific modification of VSV-G was monitored. As can be seen in Fig. 1C (lane 1, vector), when VSV-G was expressed in the absence of any 3A protein, most of the pulse-labeled VSV-G moved to the Golgi, becoming resistant to endoglycosidase H cleavage, during the 30-min chase in the presence of unlabeled amino acids. Expression of 3A protein from poliovirus 1 caused the newly synthesized VSV-G to be retained within the ER (Fig. 1C, lane 2), as has been shown previously (Doedens and Kirkegaard, 1995; Doedens et al., 1997). Expression of 3A protein from poliovirus Sabin 3, which differs from the type 1 protein only by exchange of Ser15 for Arg, also caused the labeled VSV-G protein to be retained in the ER (Fig. 1C, lane 4). However, when the FLAG-tagged version of either 3A protein was expressed, most of the VSV-G moved to the Golgi. Thus, although FLAG-tagged versions of each protein could accumulate in transfected cells, they no longer inhibited ER-to-Golgi traffic. In subsequent experiments, the ability of other picornaviral proteins to inhibit ER-to-Golgi traffic in COS-1 cells was therefore monitored by expressing an untagged version of each protein, and a FLAG-tagged version was used only to monitor expression. In each case, we made the assumption that the ability of each tagged version to accumulate in cells either reflected or underestimated the ability of the untagged version to accumulate, as was the case with the poliovirus proteins.

3A protein from coxsackievirus B3 also inhibits ER-to-Golgi traffic

We first tested whether picornaviral 3A proteins from viruses that are closely related to poliovirus had an effect on cellular protein secretion. When plasmids that encode 3A proteins from poliovirus 1, coxsackievirus B3, enterovirus 71 BrCr, and hepatitis A were transfected into COS-1 cells at 4 μg/100-mm plate, only hepatitis A 3A could be detected in an anti-FLAG immunoblot (Fig. 2B). It has been shown previously that poliovirus 3A protein can inhibit ER-to-Golgi traffic even at concentrations below the limits of detection by immunoblot (Doedens et al., 1997), and as expected, cells transfected with plasmids that encoded poliovirus type 1 3A showed little Golgi-specific modification of VSV-G protein (Fig. 2A). Similarly, coxsackievirus 3A protein expression slowed ER-to-Golgi traffic (Fig. 2A), even though no expression of the FLAG-tagged version was observed (Fig. 2B). No inhibition of ER-to-Golgi traffic was observed in cells that expressed hepatitis A virus 3A protein (Fig. 2A).

Detectable expression of FLAG-tagged 3A proteins from enterovirus 71 and human rhinovirus (HRV) 14 could only be achieved when the amount of transfected DNA was increased (Fig. 2D). Under these conditions, accumulation of FLAG-tagged 3A proteins from poliovirus 1, enterovirus 71, and rhinovirus 14 could be readily observed (Fig. 2D). Still, only the 3A proteins from poliovirus 1 and coxsackievirus B3, which remained undetectable when tagged, were able to inhibit secretion (Fig. 2C).

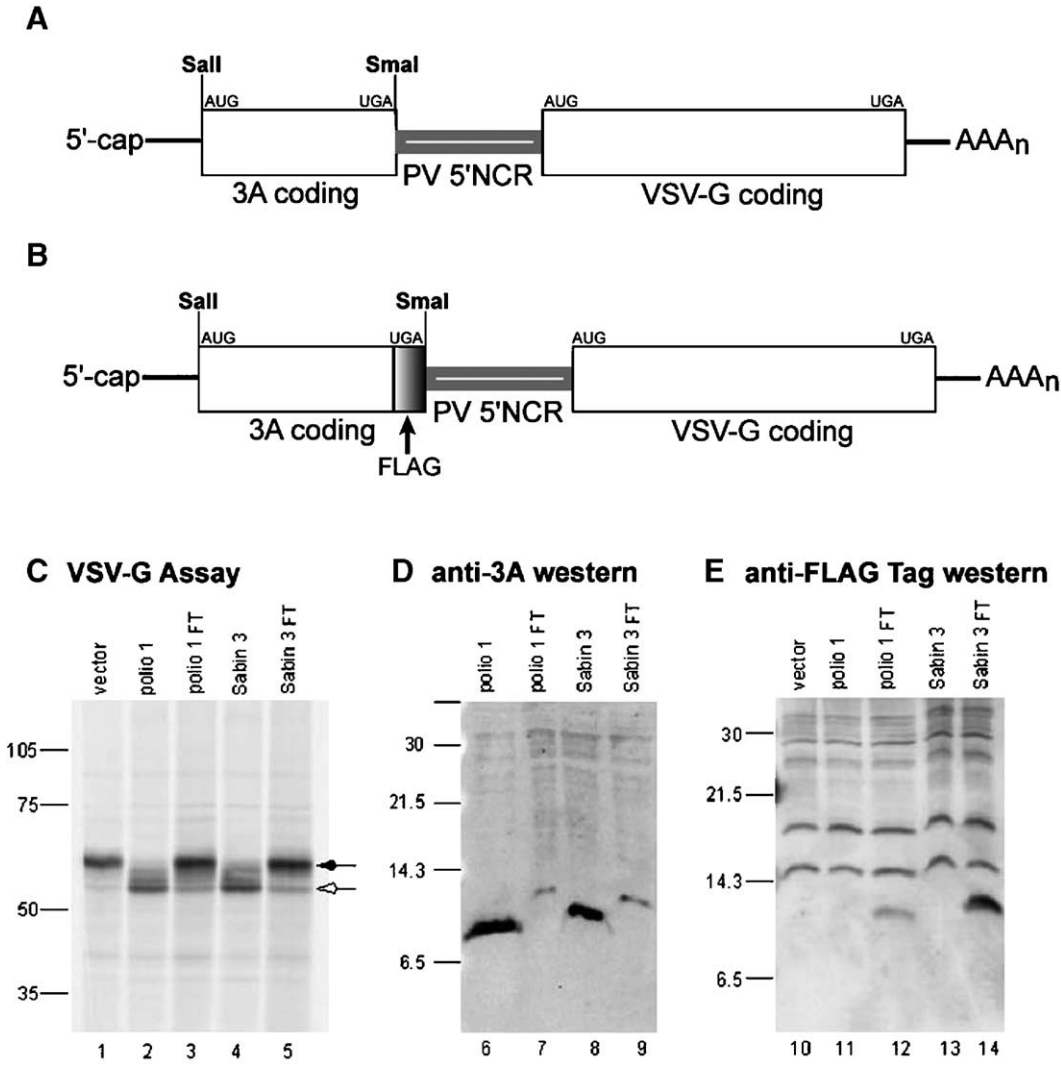

Fig. 1. Effect of tagged and untagged poliovirus 3A proteins on Golgi-specific modification of VSV-G. (A) A representation of the predicted mRNA generated by transient transfection of dicistronic plasmids used to express mutant and wild-type picornaviral 3A proteins in the first ciston, and VSV-G in the second cistron, in COS-1 cells. The dicistronic transcript is under the transcriptional control of the SV40 promoter and the VSV-G coding region in the second cistron is under the translational control of the internal ribosome binding site the poliovirus 5′ non-coding region (PV 5′NCR). (B) A diagram depicting the addition of a sequence that encodes the FLAG epitope (DYKDDDDK) at the 3′ terminus of the 3A coding region in the dicistronic plasmid shown in A. (C) VSV-G modification in the presence of untagged and tagged poliovirus 3A proteins. COS-1 cells were transfected with dicistronic plasmids that encoded 3A proteins from poliovirus Mahoney 1 (polio 1) or poliovirus Sabin type 3 (Sabin 3) without and with a C-terminal FLAG epitope as indicated. Twenty-four hours post-transfection, cells were pulse-labeled for 15 min with [^{35}S]-methionine/cysteine, chased for 30 min, harvested, and lysates were prepared. Immunoprecipitated VSV-G was cleaved with endo H, the proteins were separated by SDS–PAGE, and the results detected using a STORM phosphorimager. (D) The relative expression of untagged and tagged versions of the poliovirus 3A proteins was determined by immunoblot using an antibody that recognized both type 1 and type 3 3A proteins. (E) The relative expression of FLAG-tagged proteins was determined by immunoblot using an antibody (M2) specific for the FLAG epitope. Molecular weights (kDa) of marker proteins are indicated.

Expression and activity of 3A proteins from neurovirulent Theiler's strain GDVII, non-virulent Theiler's strain BeAn, and the O/Tai strain of foot-and-mouth disease virus (FMDV) were also monitored and tested (Monteyne et al., 1997; O'Donnell et al., 2001; Pevear et al., 1988). We also included the L protein of Theiler's virus, which is known to affect interferon secretion during infection (Fiette et al., 1995; Lin et al., 1999; Kong and Roos, 1991). The expression of FLAG-tagged versions of each of these proteins was confirmed (Fig. 2F). None of the expressed proteins, however, could be observed to alter the rate of movement of VSV-G from the ER to the Golgi (Fig. 2E, lanes 3–6).

Sequence comparisons of picornaviral 3A proteins

When the 3A sequences of poliovirus and coxsackievirus B3 were compared with those of the other tested 3A proteins, their similarities led to testable hypotheses as to the basis of the ability to inhibit ER-to-Golgi traffic (Fig. 3). Protein 3A is one of the least conserved picornaviral proteins; the 3A proteins studied here, for example, vary in length from 74 to

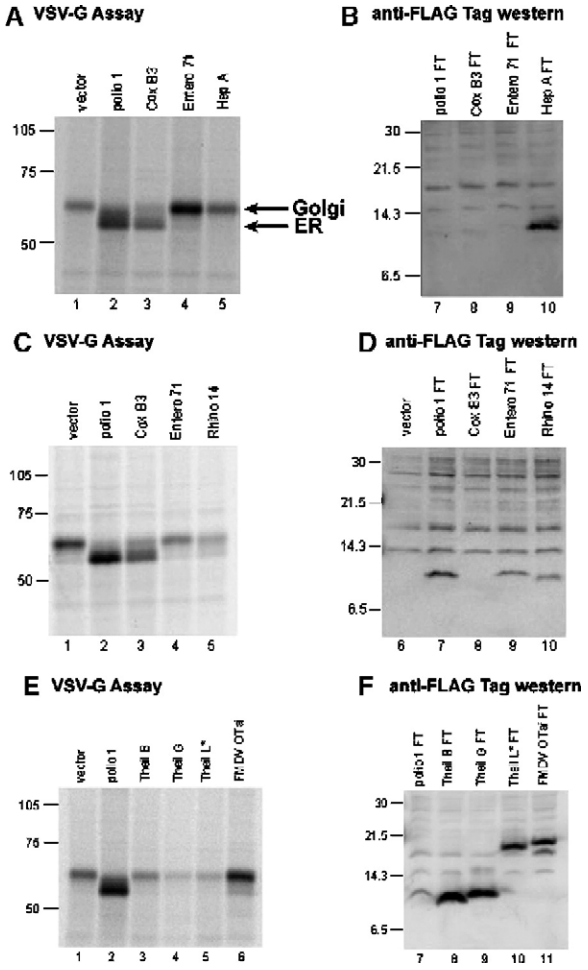

Fig. 2. Inhibition of VSV-G modification by picornaviral proteins. (A) The effect of expressing 3A proteins from poliovirus type 1 (polio 1), coxsackievirus B3 (Cox B3), enterovirus 71 (Entero 71), and hepatitis A (Hep A) viruses on Golgi-specific modification of VSV-G after transfection of 4 μg of plasmid DNA for each 100 mm plate is shown. (B) The relative accumulation of FLAG-tagged versions of the various 3A proteins tested in A was determined by immunoblot. (C) The effect of expressing several picornaviral 3A proteins, including that encoded by human rhinovirus 14 (Rhino 14) on Golgi-specific modification of VSV-G after transfection of 4 μg of plasmid DNA for each 100 mm plate is shown. (D) The relative accumulation of FLAG-tagged versions of the 3A proteins tested in C was determined by immunoblot. (E) The effect of expression of 3A proteins from poliovirus type 1, Theiler's virus BeAn (TheiI B), Theiler's virus GDVII (TheiI G), and foot-and-mouth disease virus OTai (FMDV OTai), and of the L* protein of Theiler's GDVII (TheiI L*) on Golgi-specific modification of VSV-G, after transfection of 8 μg of plasmid DNA for each 100 mm plate, is shown. (F) The relative accumulation of FLAG-tagged versions of the proteins tested in E was determined by immunoblot. Molecular weights (kDa) of marker proteins are indicated.

152 amino acids. The only sequence feature in the N-terminal half of the protein whose presence correlated with the ability to inhibit ER-to-Golgi traffic was Lys9 (Fig. 3). The structure of the first 59 amino acids of poliovirus 3A has been determined by NMR spectroscopy (Fig. 4A; Strauss et al., 2003). This structure reveals a dimer, whose interface is formed between helical hairpins present in each monomer. The amino-terminal 14 amino acids of each monomer were found to be unstructured (Fig. 4); Lys9 lies in this region and is thus a good candidate for one of the residues that mediates interaction with a cellular ligand that might lend structure to the unfolded region. The poliovirus, coxsackievirus B3, and rhinovirus 14 sequences share a stretch of three Pro residues, found at positions 16–18 in poliovirus 3A; given the known propensity of proline-containing motifs to mediate protein–protein interactions (Kay et al., 2000), these residues also seemed to be likely candidates for involvement in the inhibition of ER-to-Golgi traffic even though they were also present in the rhinovirus 14 sequence.

Mutagenesis of specific residues of poliovirus 3A abrogates secretion inhibition

To facilitate testing the activity of numerous mutant 3A proteins in transfected cells, an assay to monitor secreted alkaline phosphatase (SEAP) in tissue-culture medium and cells was employed. COS-1 cells were co-transfected with plasmids that encoded SEAP and the 3A protein of interest; thus, only secretion from transfected cells was monitored. The plasmid-encoded alkaline phosphatase activity is known to be resistant to heat inactivation, unlike endogenous alkaline phosphatase activities, so the activity measured after heat treatment represented only protein synthesized in transfected cells. In all cases, 10 μg/ml cycloheximide was

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+Poliovirus 1          -GPLQYKDLKIDIK-TSPPPECINDLLQAVDS---QEVRDYCEKKGWIVNI-TSQVQTER
+Poliovirus 3          -GPLQYKDLKIDIK-TRPPPECINDLLQAVDS---QEVRDYCEKKGWIVNI-TSQVQTER
+Coxsackievirus B3     -GPPVYREIKISVAPETPPPPAIADLLKSVDS---EAVREYCKEKGWLVPEINSTLQIEK
 Human Rhinovirus 14   -GP-VYKDLEIDVC-NTPPPECINDLLKSVDS---EEIREYCKKKWIIP--EIPTNIER
 Enterovirus 71 BrCr   -GPPKFRPIRISLE-EKPAPDAISDLLASVDS---EEVRQYCREQGWIIP--ETPTNVER
 Theiler's virus BeAn  -SPPDWEHFENILTCLRQNNAALQDQLDELQEAFAQARERSDFLSDWLKV---S-AIIFA
 Theiler's virus GDVII -SPPDWQHFENILTCLRQNNAALQDQVDELQEAFTQARERSDFLSDWLKV---S-AIIFA
 Hepatitis A HM175     -----------GIS-DDDNDSAVAEFFQSFPS---GE-PSNSKLSGFFQS---VTNHKWV
 FMDV A12              ISIPSQKSVLYFLIEKGQHEAAIEFFEGMVHDSIKEELRPLIQQTSFVKR---AFKRLKE
 FMDV O/Tai            ISIPSQKSVLYFLIEKGQHEAAIEFFEGMVHDSIKEELRPLIQQTSFVKR---AFKRLKE

+Poliovirus 1          NINRAMTILQAVTT-FAAVAGVVYVMYKLFAGHQ
+Poliovirus 3          NINRAMTILQAVTT-FAAVAGVVYVMYKLFAGHQ
+Coxsackievirus B3     HVSRAFICLQALTT-FVSVAGIIYIIYKLFAGFQ
 Human Rhinovirus 14   AMNQASMIINTILM-FVSTLGIVYVIYKLFAQTQ
 Enterovirus 71 BrCr   HLNRAVLVMQSIAT-VVAVVSLVYVIYKLFAGFQ
 Theiler's virus BeAn  GIASLSAVIKLASL-FKESIWPSPVRVELSEGEQ
 Theiler's virus GDVII GIVSLSAVIKLASK-FKESIWPSPVRVELSEGEQ
 Hepatitis A HM175     AVGAAVGILGVLVGGWFVYKHFSRKEEEPIPAE-
 FMDV A12              NFEIVALCLTLLAN-IVIMIRETRKRQKMVDDAV*63
 FMDV O/Tai            NFEVVALCLTLLAN-IVIMLRQARKRYQSVDDPL*54
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Fig. 3. Alignment of picornaviral 3A proteins. The sequences of many of the 3A proteins tested in Fig. 2 are shown in an alignment generated using the TimeLogic Decypher Clustal W multiple alignment program on the website (http://www.decypher2.stanford.edu/algo-cw/cw_ax.shtml). Hydrophobic domains thought to be involved in membrane association (Towner and Semler, 1996) have been boxed. Plus signs indicate those picornaviral 3A proteins that inhibit Golgi-specific modification of VSV-G protein. Asterisks represent amino acids that were not shown, and the numbers indicate how many amino acids the asterisks represent. Sequences were obtained from GenBank and from the published literature (Beneduce et al., 1995; Brown and Pallansch, 1995).

added concomitantly with new medium so that new SEAP was not synthesized during the time course; indeed, the total amounts of SEAP remained constant throughout the experiments (data not shown). The percentage of SEAP in the supernatant of cells co-transfected with 3A-encoding plasmid or treated with brefeldin A was greatly reduced compared to cells co-transfected with control vector DNA (Fig. 5A). In a time course of SEAP secretion from cells co-

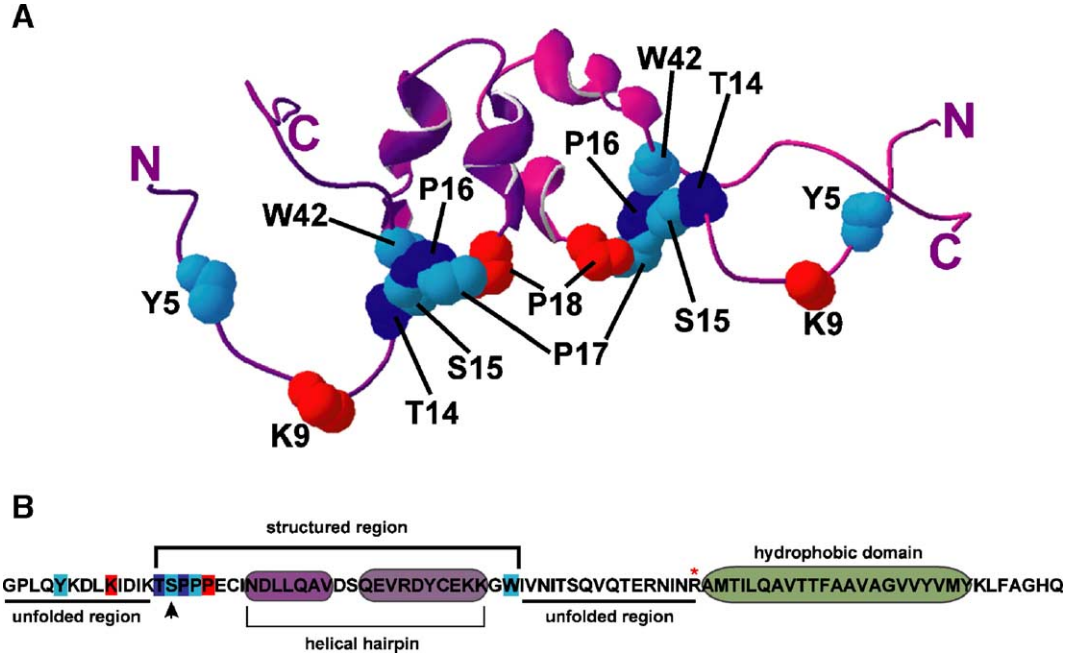

Fig. 4. Three-dimensional structure of residues 1–58 of poliovirus type 1 3A protein determined by NMR. (A) Ribbon diagram of the NMR structure of a dimer of two molecules of poliovirus type 1 3A, residues 1–58 (Strauss et al., 2003; PDB file 1NG7). The two monomers are shown in different colors (purple and pink). The locations of Tyr5, Lys9, Thr14, Ser15, Pro16, Pro17, Pro18, and Trp42 have been highlighted on both monomers. The ribbon diagram was generated using Deep View Swiss-PDB Viewer. (B) The complete amino acid sequence of poliovirus type 1 3A protein is shown. The conserved hydrophobic domain at the C-terminal end of the protein is shaded in green. The red asterisk denotes Arg58, the most C-terminal residue in the three-dimensional structure in A. Brackets indicate the regions found to be ordered in solution. The top bracket denotes the entire structured domain of 3A(1–58) and the bottom bracket flanks the amino acids involved in forming the helical hairpin, shaded in purple. The regions that are disordered in solution are underlined. The colored boxes correspond to the highlighted residues in A, which were changed by site-directed mutagenesis. The arrowhead points to the location of the serine insertion in the 3A-2 mutant protein.

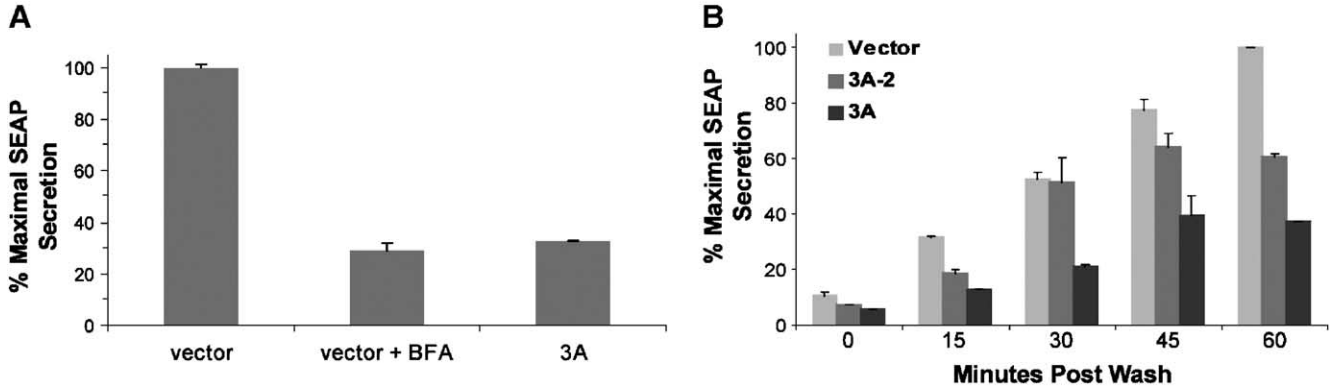

Fig. 5. Monitoring extracellularly secreted alkaline phosphatase (SEAP) as an assay of secretion from transfected cells. (A) COS-1 cells were co-transfected with a plasmid, pGG-SEAP, that encoded heat-stable-secreted alkaline phosphatase (Gene Therapy Systems, Inc.) and either a plasmid (Fig. 1) that encoded poliovirus type 1 3A protein or a vector control. Twenty-four hours after transfection, cells were washed, fresh medium containing either cycloheximide or cycloheximide and 1 mg/ml brefeldin A (BFA) was added, and incubation was continued. After 90 min, both cell lysates and supernatants were heat-treated and assayed for alkaline phosphatase activity. Maximal SEAP secretion (100%) was set at the amount of secretion in the vector control sample. The graph represents the average and standard error from three replicate samples for each condition. (B) Time course of SEAP secretion in the presence and absence of mutant and wild-type poliovirus 3A proteins. COS-1 cells were co-transfected with pGG-SEAP and either a vector control or a plasmid that expressed wild-type or 3A-2 mutant poliovirus 3A protein. Cell lysates and supernatants were collected at 0, 15, 30, 45, and 60 min, heated, and assayed for plasmid-encoded SEAP activity. Maximal SEAP secretion (100%) was set at the amount of secretion in the vector control sample at 60 min. Average values and standard error from three replicate experiments are shown.

transfected with vector DNA, DNA that expressed poliovirus 3A, or DNA that expressed 3A-2 (the mutant 3A protein that contains a Ser insertion between residues 14 and 15), the percentage of secreted SEAP was shown to increase linearly for 60 min. Again, 3A protein expression reduced secretion significantly, whereas expression of the 3A-2 mutant protein was not as effective (Fig. 5B). Therefore, the SEAP assay yielded results consistent with previous observations, and subsequent experiments to monitor the effects of new mutations were either performed as time courses or at the 30-min time point, well within the linear range of the SEAP assay.

The first mutations introduced into the 3A coding region were T14A/S15A and P16A, substituting Ala residues for both Thr14 and Ser15 or for Pro16, respectively. These residues were chosen because they flank the serine insertion site of the 3A-2 mutation (Bernstein and Baltimore, 1988; Doedens et al., 1997), a mutation that destroys the ability of 3A protein to inhibit ER-to-Golgi traffic but has little effect on viral viability (Dodd et al., 2001; Doedens et al., 1997). Immunoblot analysis of the transfected cell lysates demonstrated that wild-type and mutant 3A proteins were present at similar abundance in transfected cells (Fig. 6A, lower panel). Interestingly, the T14/S15A and P16A proteins retained the ability to inhibit ER-to-Golgi traffic. Therefore, although a single Ser insertion in this region destroyed the activity, alanine substitutions for the neighboring residues did not.

The next region examined for residues critical to the inhibition of secretion was the triple-proline motif at amino acid positions 16–18: additional mutant 3A-encoding plasmids P17A, P18A, P16A/P17A, and P16A/P17A/P18A were shown to express 3A protein as abundantly as the wild-type plasmid (Fig. 6B). Of these, only two mutant 3A proteins, P18A and P16A/P17A/P18A, disrupted the ability of 3A protein to inhibit cellular protein secretion (Fig. 6B). Therefore, of the three Pro residues, only Pro18 is indispensable for protein secretion inhibition activity of 3A. This sequence requirement for a single Pro residue could be due to a crucial role in global folding, direct interaction with a ligand, or a structural requirement such as demarcating the natively unstructured N-terminal region.

Another mutation, W42F, was introduced because it is known to eliminate viral viability (Strauss et al., 2003). Trp42 is highly conserved among the picornaviral 3A proteins tested here (Fig. 3) and was therefore not likely to play a role in the inhibition of ER-to-Golgi traffic. As expected, the W42F mutation had little effect on the ability of 3A protein to inhibit protein secretion (Fig. 6B), again highlighting the fact that 3A protein has multiple functions, some of which are required for viral viability, and some of which, such as the effect on host protein secretion, do not correlate with growth in tissue culture but are likely to affect pathogenesis in infected hosts.

Of the most N-terminal sequences, Lys9 is conserved only in poliovirus and coxsackievirus 3A proteins, and Tyr5 in poliovirus, coxsackievirus, and rhinovirus 14 3A proteins (Fig. 3). As shown in Fig. 6C, the Y5W mutation did not disrupt the protein's ability to inhibit cellular protein secretion, whereas the K9G mutant 3A protein was no longer able to inhibit secretion, displaying a defect similar to that of 3A-2. Neither the Y5W nor the K9G mutations interfered with 3A protein expression (Fig. 6C).

Mutations that abrogate ER-to-Golgi traffic create loss-of-function alleles

It is often assumed that deleterious mutations simply disrupt the normal function of the wild-type protein. We

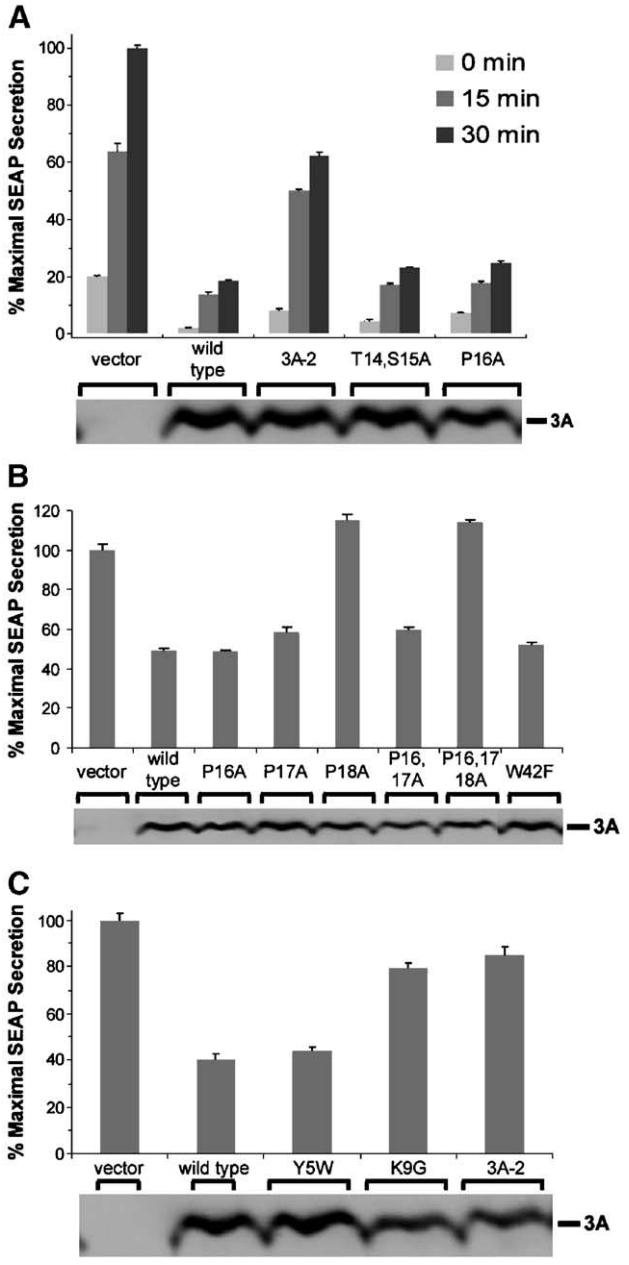

Fig. 6. Effect of site-directed mutations on 3A-mediated inhibition of protein secretion. (A) COS-1 cells were co-transfected with pGG-SEAP and either vector DNA or DNA that encoded wild-type 3A protein or mutant 3A proteins 3A-2, T14A/S15A, or P16A are shown. Samples were collected at 0, 15, and 30 min post-wash; maximal secretion (100%) was set at the 30-min time point for the vector sample; data from triplicate experiments are shown. Below the graph, an immunoblot of an SDS gel that displayed proteins from the 30-min time points, probed with anti-3A antibody, is shown. (B) The effect of the indicated mutations was determined as in A. Media and cells from the 30-min time point were collected and assayed for heat-stable alkaline phosphatase activity; data from triplicate experiments are shown. Below the graph, an immunoblot using anti-3A antibody is shown. (C) The effects of mutations Y5W and K9G on SEAP secretion and 3A protein accumulation were compared to the effect of the 3A-2 mutation as in B; data from six replicate experiments are shown.

would certainly like to interpret the effects of the K9G, P18A, and 3A-2 changes as loss-of-function mutations. However, dominant, gain-of-function alleles also exist which, for example, form unproductive oligomeric structures or target critical components inappropriately. To test whether several 3A alleles that do not inhibit ER-to-Golgi traffic [poliovirus 3A-2, FLAG-tagged poliovirus 3A (3A.FT) and FLAG-tagged enterovirus 71 3A.FT] were recessive or dominant in combination with wild-type poliovirus 3A with respect to inhibition of cellular protein secretion, the amounts of co-expressed wild-type and mutant 3A protein were varied reciprocally. As a control, the amount of transfected wild-type 3A plasmid was varied, while the total amount of DNA transfected was kept constant via the addition of vector DNA (Fig. 7A). SEAP assays revealed that cellular protein secretion was still substantially inhibited even when wild-type 3A protein was not detectable by immunoblot (Fig. 7A). When any of the variant 3A-encoding plasmids was titrated into the transfection mixtures, the effects on cellular protein secretion were indistinguishable from the addition of vector DNA (Figs. 7B, C, and D). In the case of 3A-2, the expression of mutant 3A protein could be verified by immunoblot (Fig. 7B). Therefore, we conclude that the 3A-2, 3A.FT, and enterovirus 3A.FT-encoding plasmids are recessive, making it likely that the encoded mutant proteins have lost the wild-type function responsible for inhibiting ER-to-Golgi traffic.

3A protein from rhinovirus 14 can be mutagenized to inhibit protein secretion

Lys9, present in the N-terminal region of poliovirus and coxsackievirus 3A, was shown to be crucial to the ability of poliovirus 3A to inhibit host protein secretion (Fig. 6C). In the 3A sequence of rhinovirus 14, which does not inhibit host protein secretion, the corresponding residue is Glu8; the numbering is altered because the rhinovirus 3A sequence also lacks Gln4 relative to the poliovirus sequence and Pro3 relative to the coxsackievirus sequence (Fig. 3). To test whether the ability to inhibit host protein secretion could be conferred to rhinovirus 14 3A protein by changes in the extreme N-terminus of the protein, the mutations E8K and P3i (an insertion of a Pro residue at position 3) were introduced. As shown in Fig. 8, the E8K mutation conferred a partial ability to inhibit protein secretion to the rhinovirus 3A protein, an effect that may have been increased somewhat by the presence of the P3i mutation as well. Therefore, small sequence changes to the rhinovirus 14 3A sequence can confer increased ability to inhibit host protein secretion.

Discussion

The benefit conferred to poliovirus by the ability of 3A protein to inhibit cellular ER-to-Golgi traffic is not yet known. It appears that the most simple possibility, that the inhibition of protein secretion results from the role of 3A in

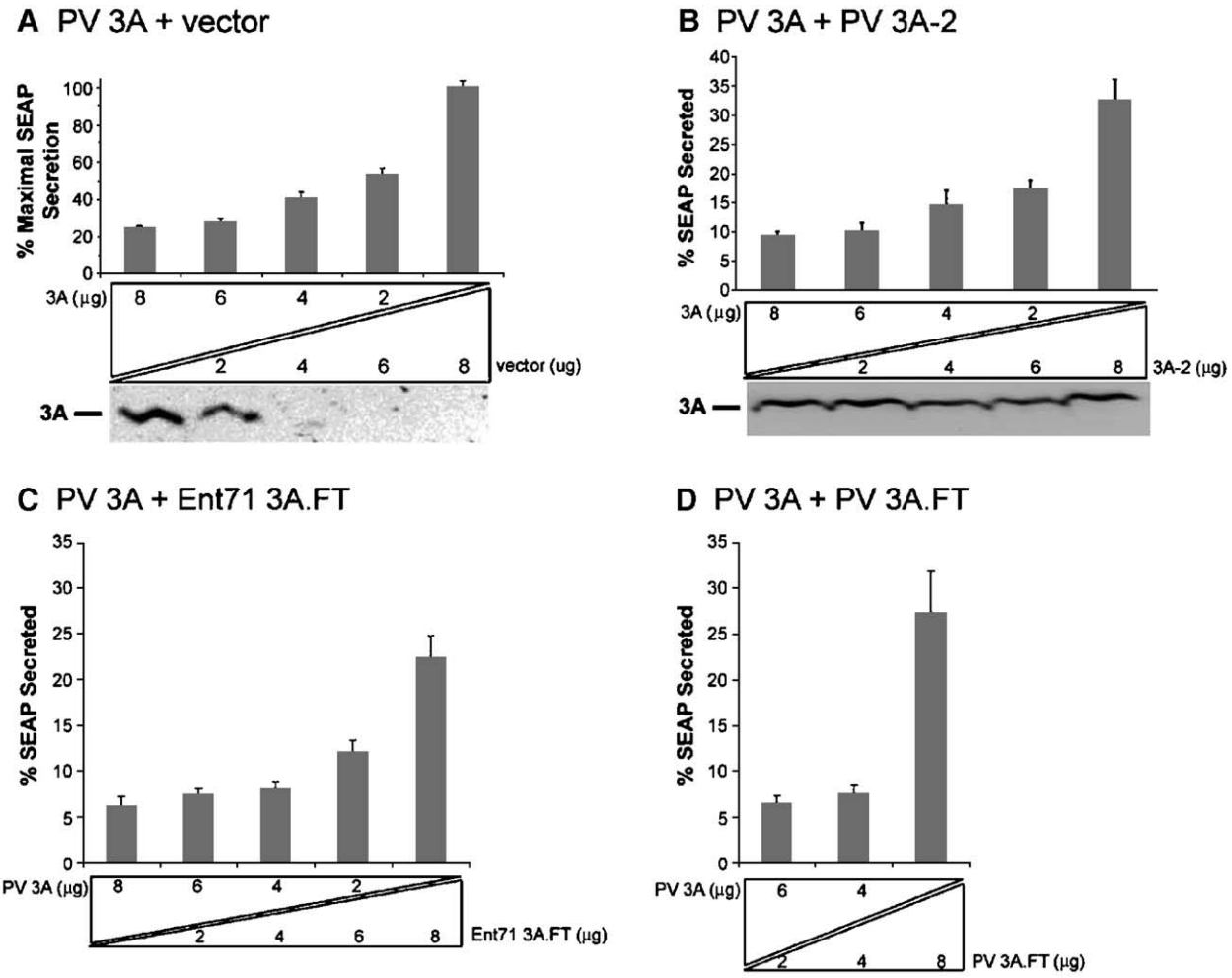

Fig. 7. Co-transfection of wild-type and inhibition-deficient 3A mutant proteins. (A) COS cells were co-transfected with pGG-SEAP and varying mixtures of 3A-expressing plasmid and vector as indicated. Media and cells were collected at the 30-min time point as in Fig. 6B. Maximal secretion (100%) was set as percentage of SEAP secretion in the 30-min vector-alone sample. Below the graph, an immunoblot using anti-3A antibody is shown. (B) The ability of decreasing amounts of wild-type 3A protein to inhibit SEAP secretion in the presence of increasing amounts of DNA encoding 3A-2 mutant protein is shown. An immunoblot using anti-3A antibody is shown below the graph. (C) The ability of decreasing amounts of wild-type 3A protein to inhibit SEAP secretion in the presence of increasing amounts of FLAG-tagged enterovirus 71 3A protein (Ent71 3A.FT) or (D) FLAG-tagged poliovirus 3A protein is shown. Averages and standard error from three replicate samples are indicated.

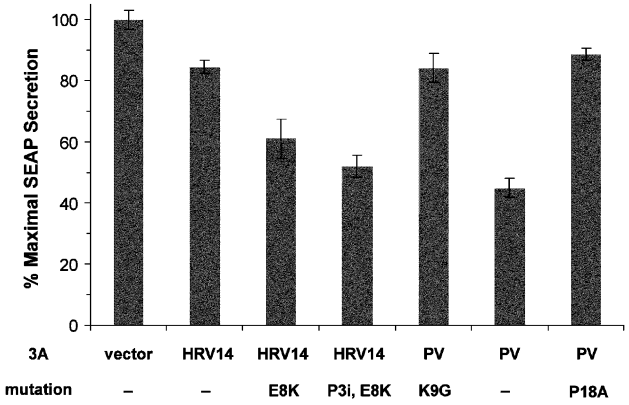

Fig. 8. Effect of site-directed mutations on the ability of 3A from human rhinovirus 14 to inhibit protein secretion. COS-1 cells were co-transfected with pGG-SEAP and either vector DNA, DNA that encoded wild-type 3A protein from human rhinovirus (HRV) 14, poliovirus type 1 (PV), or mutant HRV 14 and poliovirus 3A proteins as indicated. Samples were collected at 30 min post-wash; maximal secretion (100%) was set for the vector sample; data from triplicate experiments are shown.

the assembly of viral RNA replication complexes on intracellular membranes, is not likely because the functions of 3A in viral RNA replication and in inhibiting protein secretion can be genetically separated (Table 1). As reported previously, a mutant poliovirus, 3A-2, has been isolated that does not display a significant growth defect in tissue culture at 37 °C and induces the membranous vesicles characteristic of wild-type poliovirus infection (Bernstein and Baltimore, 1988; Dodd et al., 2001) but fails to inhibit host protein secretion (Doedens et al., 1997; Dodd et al., 2001). Similarly, mutations in the 3A coding region have been identified that destroy viral viability but not the ability of 3A protein expressed in isolation to inhibit host protein secretion (Doedens et al., 1997; Strauss et al., 2003; this work). It will be interesting to learn whether the known effects of type I interferons on limiting viral growth in a mouse model for poliovirus infection (Ida-Hosonuma et al., 2005) will be exacerbated for mutant viruses that allow greater amounts of type I interferon secretion.

The ability of 3A proteins to inhibit cellular protein secretion is not conserved among picornaviruses. In fact, only the 3A proteins from closely related poliovirus serotype 1, poliovirus serotype 3, and coxsackievirus B3 were found to slow ER-to-Golgi traffic in COS-1 cells (Fig. 2). In these studies, the inhibition of ER-to-Golgi traffic observed for poliovirus 3A protein was found to be limited to poliovirus and coxsackievirus B3 3A proteins. However, to conclude that the 3A proteins encoded by rhinovirus 14, hepatitis A, foot-and-mouth disease virus, and Theiler's virus do not inhibit ER-to-Golgi traffic, one must assume that each of these proteins was successfully expressed, as suggested by the detectable expression of their FLAG-tagged versions. Specific antibodies were not available to confirm expression of the untagged versions, but the expression of VSV-G protein from the second cistron of the dicistronic mRNAs confirms in each case that the mRNAs were transcribed and stable. We were unable to express the untagged or tagged versions in cells from bovine or porcine hosts. Therefore, the conclusion that the 3A proteins from the picornaviruses tested besides poliovirus and coxsackievirus B3 must be qualified by these considerations.

It has been reported recently that the 3A protein from foot-and-mouth disease virus does not inhibit protein secretion in Vero cells using an immunofluorescence assay (Moffat et al., 2005). The authors suggest that, since the 2BC protein encoded by FMDV inhibits protein secretion, this activity of 3A protein in isolation is not needed. For other picornaviruses, it is also possible that their different growth strategies make inhibiting host protein secretion, and the reduction in inflammation, immune response, and cell–cell communication that should accompany it, less desirable. Perhaps, for viruses such as rhinovirus, for which inflammation of the infected tissues enhances viral spread, there is even positive selection for viruses that allow inflammatory responses.

Table 1
Viability of viruses and ability of mutant 3A proteins to inhibit host protein secretion for viruses that contain mutations in 3A coding region

	Secretion inhibition	Virus viability	Reference
Wild-type	+	+	Doedens et al., 1997
3A-2	–	+	Doedens et al., 1997; Bernstein and Baltimore, 1988
W42F	+	–	This work; Strauss et al., 2003
T67I	+	–	Doedens et al., 1997

Alignment of the 3A proteins that are capable of inhibiting ER-to-Golgi traffic (Fig. 3) revealed one candidate residue, Lys9, in the amino-terminal half of the 3A protein that correlated with this ability. Indeed, the K9G mutation was one of the few point mutations tested that destroyed the inhibitory activity of poliovirus 3A protein (Fig. 6C). Other mutations that interfered with the ability of 3A to inhibit protein secretion were P18A (Fig. 6B) and 3A-2 (Doedens et al., 1997), an insertion of a Ser residue between Thr14 and Ser15. Lys9 and Pro18 are shown in red on the structure of the 3A(1–58) dimer in Fig. 4; we suggest that these residues define part of the binding site of an as-yet-unidentified ligand to which poliovirus and coxsackievirus 3A proteins bind to effect the inhibition of protein secretion. Consistent with the crucial role of Lys9, altering the rhinovirus 3A protein sequence to contain a Lys residue at the homologous position (the E8K mutation) conferred a partial ability to block host protein secretion (Fig. 8).

The ordering of unstructured protein domains upon ligand binding has been found in many instances in the protein secretory pathway (reviewed in Dafforn and Smith, 2004). One example is the formation of the SNARE complex. The components Sec9 and Snc1, as well as the N-and C-terminal domains of Sso1, are unstructured in isolation, but become structured upon formation of a Sec9–Snc-1–Sso1 ternary complex (Fiebig et al., 1999). The HIV Nef protein is an example of a viral protein that contains unstructured regions that are involved in regulating its membrane-binding ability as well as its interactions with at least 30 different binding partners (Arold and Baur, 2001). Similarly, it is likely that the unstructured amino termini of picornaviral 3A proteins may have different partners to correspond to the roles of these proteins in RNA replication, membrane rearrangements, and inhibition of the host secretory pathway.

Materials and methods

Cells

COS-1 cells were cultured as monolayers in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol)

calf serum, 100 units of penicillin/ml, and 100 units of streptomycin/ml at 37 °C and 5% CO_2.

Plasmids and transfections

cDNAs for rhinovirus 14 (from W. Lee, University of Wisconsin, Madison, WI), Theiler's virus GDVII and BeAn (from H. Lipton, Northwestern University, IL), Hepatitis A HM175 (from G. Morace, Instituto Superiore di Sanita, Rome, Italy), and FMDV A12 and Otai (from P. Mason, Plum Island Animal Disease Center, New York) were used to amplify the 3A coding sequences by PCR. Poliovirus 3 (from Y. Maldonado, Stanford University) and enterovirus 71 BrCr (from B. Brown, CDC, Atlanta, GA) were obtained as RNA, and the 3A regions were amplified by RT-PCR. Oligonucleotide primers were designed to introduce a *Sal*I site and an AUG codon at the 5′end and a stop codon followed by a *Sma*I site at the 3′end of each 3A protein coding region. *Sal*I- and *Sma*I-digested PCR products were inserted into a modified version of pLink (Doedens and Kirkegaard, 1995), pNLink (Figs. 1A–B). pNLink is the same as pLink except that one internal *Sal*I site was destroyed. pNLink that lacks a coding region in the first cistron was used as the vector control. Mutations were introduced into the poliovirus 3A coding region by performing site-directed mutagenesis using PCR. All clones were sequenced to verify that the amplified regions did not contain unintended mutations.

The pGeneGrip-Secreted Alkaline Phosphatase (pGG-SEAP) plasmid was purchased from Gene Therapy Systems, Inc. (San Diego, CA). It consists of the coding region for a modified alkaline phosphatase, modified to be secreted from the transfected cells, under the transcriptional control of a CMV promoter. COS-1 cells were transfected in 10-cm tissue culture dishes using Lipofectamine Plus reagents as described by the manufacturer, with 20 μl of Plus reagent and 30 μl of Lipofectamine reagent (Invitrogen Life Technologies, Carlsbad, CA). pNLink-based plasmids (8 μg per transfection) and pGG-SEAP plasmid (2 μg per transfection) were used for SEAP assay transfections. Either 4 or 8 μg of pNLink-based plasmids was used for VSV-G modification assay transfections.

VSV-G Golgi-specific modification assay

The rate of VSV-G modification in the Golgi was monitored essentially as described previously, where it was shown that the inhibition of Golgi-specific modification by wild-type poliovirus 3A protein accompanied the retention of cargo in the endoplasmic reticulum and that a 30-min time point reflected the initial rate of Golgi-specific modification (Doedens and Kirkegaard, 1995; Doedens et al., 1997). Twenty-four hours post-transfection, cells in 100-mm dishes were washed with PBS and incubated for 15 min at 37 °C in DMEM lacking methionine and cysteine (Gibco) supplemented with [^{35}S]-methionine/cysteine (Expres^{35}S^{35}S label, New England Nuclear). Cells were then washed once with DMEM that contained unlabeled methionine and cysteine and incubated in the same medium for 30 min at 37 °C. Cells were then washed with ice-cold PBS, harvested, and centrifuged at 300 × *g* for 5 min. The supernatants were removed, and the cell pellets were lysed by resuspending in RSB-NP40 (10 mM Tris–HCl pH 7.5, 10 mM NaCl, 1.5 mM $MgCl_2$, 1% NP40). Nuclei and insoluble debris were pelleted by centrifugation at 2000 × *g* for 10 min. Supernatants were then either stored at −80 °C or subjected immediately to immunoprecipitation.

To immunoprecipitate VSV-G protein, 9.8 μg monoclonal anti-VSV-G antibody (clone P5D4, Sigma, St. Louis, MO) was added and the samples gently rotated at 4 °C for 2 h. Protein G agarose beads (25 μl of a 50% solution; Gibco BRL) were added and again the samples were gently rotated at 4 °C for 2 h, upon which the agarose beads were collected, washed once in RSB-NP40, once in RSB-NP40 that contained an additional 500 mM NaCl, and once more in RSB-NP40. The immune complexes were released from the beads by adding 20 μl of a solution that contained 0.1 M NaOAc (pH 5.5), 0.5% SDS, and 0.1 M β-mercaptoethanol, heating to 95 °C for 5 min. Endoglycosidase H (150,000 units; New England Biolabs; Beverly MA) was added, and samples were incubated overnight at 37 °C. Proteins were separated by electrophoresis on a 10% SDS–PAGE gel, dried on a gel dryer for 2 h at 80 °C, and exposure to a phosphorimager screen. Samples were viewed on a phosphorimager (Molecular Dynamics, Sunnyvale, CA) and analyzed with ImageQuant software.

SDS–PAGE gels and immunoblotting

To quantify the amount of accumulated 3A proteins, proteins from COS-1-transfected lysates were separated by tricine–SDS–PAGE using a 4% stacking and a 16.5% running gel (Schagger and von Jagow, 1987), and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA) for 1 h at 800 mA using a Hoeffer tank transfer system (Hoeffer Pharmacia Biotech, San Francisco, CA). The efficiency of transfer was verified using prestained molecular weight markers (Amersham).

After transfer was complete, the membranes were dipped in methanol and dried at room temperature. Membranes were treated by incubation from 1 to 12 h at 4 °C in PBS containing 0.1% Tween (PBS-T) and either 2% BSA or 5% nonfat dry milk. Primary antibody was diluted in blocking buffer or PBS-T, anti-3A at 1:10, M2 anti-FLAG (Sigma) at 1:1000, and added to the membrane for either 1 h at room temperature or overnight at 4 °C. Next, the membranes were washed four times in PBS-T for 10 min each time, followed by incubation with alkaline phosphatase-conjugated donkey-anti-mouse secondary antibody diluted 1:5000 in PBS-T for 1 h at room temperature. Blots were developed in ECF reagent (Amersham) for 5 min, viewed on a phosphorimager

(Molecular Dynamics), and analyzed with ImageQuant software.

Alkaline phosphatase assays

From each plate of transfected cells, 0.5 ml of medium (0.05 of the total volume) was collected and stored on ice. Collected cells were washed once in ice-cold PBS, scraped into 1-ml ice-cold PBS using a rubber policeman, pelleted at 500 × *g* for 4 min at 4 °C, and lysed in 100 μl RSB-NP40 buffer containing 1 mM PMSF. Lysates were incubated on ice for 20 min and centrifuged at 20,000 × *g* for 20 min at 4 °C; the supernatants were collected. Supernatants and lysates from untransfected cells were also prepared and used as background controls. All samples were heat-inactivated at 65 °C for 30 min and cooled to room temperature. Samples were diluted appropriately to final volumes of 20 μl and added to a well of a flat-bottom 96-well plate along with 10 μl of 0.05% Zwittergent 3-14 (CalBiochem, EMD Biosciences, Inc., San Diego, CA). Substrate solution was made by dissolving para-nitrophenylphosphate (pNPP) Sigma 104 Phosphatase Substrate (Sigma, St. Louis, MO) to 1 mg/ml in buffer containing 1 mM $MgCl_2$ and 100 mM diethanolamine (pH 9.8). Substrate solution and samples were prewarmed to 30 °C prior to mixing. Substrate solution (200 μl) was added to each sample. After 15 min of incubation at 30 °C, reactions were stopped by the addition of 50 μl of 3 N NaOH. Absorbance was measured at 405 nm on a plate reader.

Molecular modeling

Modeling was performed with the Swiss PDB Viewer (http://www.expasy.ch/spdbv) (Guex and Peitsch, 1997). Coordinates for 3A(1–58) were obtained from Debra Wuttke (University of Colorado at Boulder) and can be obtained from the NCBI library under PDB file 1NG7 (Strauss et al., 2003).

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